Distribution of Neuropeptide Receptors

New Views of Peptidergic Neurotransmission Made Possible by Antibodies to Opioid Receptors[a]

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Chemical neurotransmission can be categorized by its major features. Temporally, one can compare fast versus slow neurotransmission; the former occurs over a time course of milliseconds whereas the latter ranges from seconds to minutes in time course. Chemical neurotransmission can also be divided by the structural arrangement of its neurotransmitter delivery system, that is, synaptic versus nonsynaptic arrangements. Synaptic transmission is a highly compartmentalized form of intercellular communication in that the site of release of a transmitter is separated by only 30 nm from receptors on the postsynaptic membrane. Nonsynaptic neurotransmission, in contrast, is less confined in that a transmitter is released and must diffuse through a significantly greater volume of extracellular space than the synaptic cleft before encountering its receptor.[1,2]

Nonsynaptic neurotransmission is now thought to be an important phenomenon because histochemical studies of the distribution of neurotransmitters and their receptors have repeatedly discovered widespread "mismatches" in the spatial relationships of transmitters and receptors.[3,4] Because of the limitations of histochemical methods, these assessments of the mismatch problem have been indirect and inferential. Developments outlined below have, for the first time, enabled the direct examination of the spatial relationship between a neuropeptide and its receptor.[5–9]

Peptidergic neurotransmission appears to be accomplished by slow, nonsynaptic mechanisms.[10,11] The cloning of receptors for neuropeptides has contributed impor-

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tantly to our understanding of signal transduction for this class of neurotransmitters. The first of the neuropeptide receptors to be cloned were the tachykinin receptors[12,13] and the receptor for neurotensin,[14] but several other families have been subsequently cloned. Cloning of these receptors has been satisfying in several regards, not the least of which was to establish that they are indeed members of the seven-transmembrane, G-protein-coupled family of receptors.

In addition, the cloning of neuropeptide receptors has allowed significant advances in our understanding of the deployment of these receptors by neurons to mediate the actions of neuropeptides. First, *in situ* hybridization studies have permitted the identification of the cells that express a given, cloned receptor.[15] Second, the ability to produce antibodies to portions of the predicted primary structure of cloned receptors[5–8,16] and to use these antibodies in immunocytochemical studies has allowed a determination of the targeting of the receptor to pre- or postsynaptic sites and also of the spatial relationship between the receptor and nerve terminals that contain its ligand.[5–9] Such findings have helped to advance our understanding of the nature of peptidergic neurotransmission; this point serves as the focus of this review. Because of the relative infancy of this field of inquiry, most of the data reviewed herein will be from the family of opioid receptors, which have been the focus of studies in the authors' laboratories.

TECHNICAL CONSIDERATIONS

Prior to the cloning of receptors for neuropeptides, the various methods of ligand-binding autoradiography provided the only histochemical method capable of revealing the distribution of these receptors.[17–20] Much was learned concerning the regional occurrence of neuropeptide receptors by autoradiography, including the conclusion that in many instances a spatial mismatch occurs between a given receptor and nerve terminals containing a ligand for this receptor. Although some investigators resorted to heroic measures,[21,22] in general, the autoradiographic methods were limited in several aspects: First, it was difficult to draw conclusions directly concerning the pre- versus postsynaptic nature of a receptor. Second, limitations in resolution precluded determination of whether a localized receptor was in the plasma membrane or in an intracellular compartment. Third, ligand-binding autoradiographic methods were not capable of revealing the distribution of receptors per se, but rather the distribution of binding sites. Although it was possible to control incubation conditions so as to maximize the likelihood that binding sites were indeed associated with receptors, it was unclear whether the correlation was perfect.

The cloning of neuropeptide receptors made possible the application of techniques that could complement and at least partially overcome the limitations of ligand-binding autoradiography. That is, knowledge of the sequence of mRNA encoding a receptor makes possible the localization of transcripts for that receptor by *in situ* hybridization, while the predicted primary structure of the receptor protein allows the development of antibodies capable of recognizing the receptor protein using immunocytochemical methods.

In Situ *Hybridization*

In situ hybridization methods for localization of neuropeptide receptor mRNA are not different than those for other transcripts, except that the abundance of most

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TABLE 1. Antipeptide Antisera against Opioid Receptors and Opioid Peptides

	Species Immunized	Amino Acid Numbers (species)	Amino Acid Sequences[a]	Reference
Receptors				
δ-Opioid receptor (DOR1)	Rabbit/rat	3–17 (mouse)	LVPSARAELQSSPLV[b]	Dado *et al.*[7]
		30–46 (mouse)	AGANASGSPGARSASSL[b]	Arvidsson *et al.*[5]
		103–120 (mouse)	PFQSAKYLMETWPFGELL[b]	
μ-Opioid receptor (MOR1)	Rabbit	384–398 (rat)	NHQLENLEAETAPLP[b]	Arvidsson *et al.*[6]
κ-Opioid receptor (KOR1)	Rabbit	366–380 (rat)	DPASMRDVGGMNKPV[b]	Riedl *et al.*[41]
Peptides				
Leu-enkephalin	Mouse	1–5 (mammals)	YGGFL	Cuello *et al.*[51]
Dynorphin A1–8	Rabbit	1–8 (porcine)	YGGFLRRI	Weber *et al.*[52]
Preprodynorphin C-peptide	Guinea pig	235–248 (rat)[c]	SQENPNTYSEDLDV[b]	Arvidsson *et al.*[41]

[a]Underscoring indicates the epitope recognized by the antisera.
[b]Cross-species alignment of these are shown in TABLE 2.
[c]Sequence as reported by Douglass *et al.*[53]

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TABLE 2. Cross-species Alignment of Sequences Used for Generation of Antipeptide Antisera[a]

δ-Opioid receptor (DOR1)			
Mouse	3-LVPSARAELQSSPLV-17	30-AGANASGSPGARSASSL-46	103-PFQSAKYLMETWPFGELL-120
Rat	PVPSARAELQFSLLA	ASANASGSPGARSASSL	PFQSAKYLMETWPFGELL
Human	PAPSAGAELQPPLFA	AGANASGPPGPGSASSL	PFQSAKYLMETWPFGELL
μ-Opioid receptor (MOR1)			
Rat	384-NHQLENLEAETAPLP-398		
Mouse	-xENLEAETAPLP		
Human	NHQLENLEAETAPLP		
κ-Opioid receptor (KOR1)			
Rat	366-DPASMRDVGGMNKPV-380		
Mouse	DPASMRDVGGMNKPV		
Guinea pig	DPAYMRNVDGVNKPV		
Human	DPAYLRDIDGMNKPV		
Preprodynorphin C-peptide (ppDYN)			
Rat	235-SQENPNTYSEDLDV-248		
Porcine	SQEDPNAYYEELFDV		
Human	SQEDPNAYSGELF		

[a]Underscoring indicates differences from the first sequence in each series.

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of which mediate nociception. Early studies on the localization of opioid receptors by autoradiographic methods determined this to be a region highly enriched in opioid receptors[18,19,42] and that a significant fraction of these receptors were presynaptic in nature and resided on terminals of primary afferent neurons.[42] Localization of DOR1 by immunocytochemical means confirmed this idea (FIG. 1C and D)[5,7] and established that this receptor is exclusively presynaptic in this and many other regions of the CNS. Localization of MOR1 by immunocytochemistry (FIG. 1A and B) has demonstrated that this receptor is frequently presynaptic in the superficial dorsal horn, is expressed by primary afferent neurons, and coexists in some of these neurons with DOR1-ir.[6] However, MOR1-ir is also found in membranes of neuronal perikarya and dendrites within the superficial dorsal horn (FIG. 1B). The cloned KOR1 is similar to the DOR1 in that it is prominent in a subset of small- and intermediate-diameter perikarya in dorsal root ganglia (not shown) and in nerve terminals in the superficial dorsal horn (FIG. 1E and F).[41] It also resembles MOR1 in that it is found as a postsynaptic receptor on some neurons in the superficial dorsal horn (FIG. 1F). Interestingly, both enkephalin (ENK)- and preprodynorphin (ppDYN)-ir are found in axons and terminals in close proximity to DOR1, MOR1, and KOR1 (FIG. 1B, B′; D, D′; and F, F′). Although these represent close appositions, it does not imply that these relations are truly synaptic, because, in the case of the presynaptic opioid receptors on CGRP-positive primary afferent terminals, little evidence exists that such terminals receive axo-axonic synapses.[43] Furthermore, these opioid receptors are unlikely to be autoreceptors, because coexistence of any of these pairs of opioid ligands and receptors is very rare.

Locus Coeruleus

Neurons of the locus coeruleus express MOR1 mRNA and target the resultant protein to the plasma membrane of their cell bodies and dendrites (FIG. 2A and B[6]). Although the density of MOR1-ir is great in the neuropil of locus coeruleus, ENK-ir is present in nerve fibers and terminals in substantially smaller quantities than MOR1-ir (FIG. 2B and B′). DOR1-ir is also present in locus coeruleus, but, as in the spinal cord, is restricted to nerve fibers and terminals (FIG. 2C and D) where it presumably acts in a presynaptic manner. Again, as in spinal cord, ENK-ir is present in axons and terminals in close proximity to DOR1-ir (FIG. 2D and D′), but DOR1 is unlikely to be an autoreceptor because DOR1- and ENK-ir were not found to coexist.

Interpeduncular Nucleus

Nerve fibers and teriminals in the interpeduncular nuclei display complementary patterns of ENK- and DOR1-ir (FIG. 3A and B; Lee *et al.,* in preparation). The lateral portion of the nucleus contains a dense network of axons with DOR1-ir and almost no ENK-ir (FIG. 3A), whereas the converse relationship exists in the neighboring region (FIG. 3B). Similar, complementary patterns of staining have been observed in many brain regions, and they suggest that this orderly, spatial mismatch of opioid peptides and their receptors reflects a form of internuclear, nonsynaptic neurotransmission.

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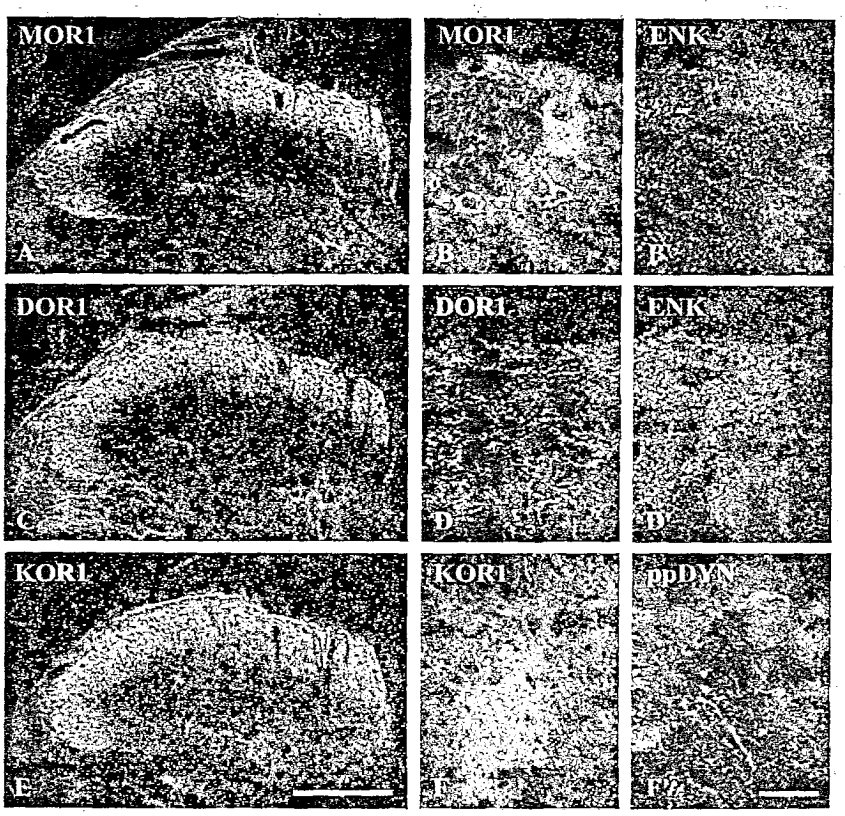

FIGURE 1. Distribution of opioid receptors and their putative endogenous ligands in the superficial dorsal horn as viewed with laser scanning confocal microscopy. Immunofluorescence images of coronal sections of the adult rat dorsal horn single stained with rabbit anti-μ-opioid receptor (MOR1; **A**) or rabbit anti-δ-opioid receptor (DOR1; **C**) or rabbit anti-κ-opioid receptor (KOR1; **E**) antisera. The opioid receptor immunoreactivity was visualized with cyanine 3.18-conjugated secondary antibodies. Note dense staining in the superficial laminae of the dorsal horn for all three opioid receptors. Two-color immunofluorescence experiments were done in order to examine the spatial distribution of opioid receptors in relation to endogenous ligands. Sections were simultaneously incubated with a mixture of rabbit anti-MOR1 (**B**) and mouse anti-enkephalin (ENK, **B′**) or rabbit anti-DOR1 (**D**) and mouse anti-ENK (**D′**) or rabbit anti-KOR1 (**F**) and guinea-pig anti-preprodynorphin (ppDYN, a marker for dynorphin; **F′**) antisera. After incubation the sections were stained with lissamine rhodamine- and fluorescein-conjugated secondary antibodies. Complementary but not overlapping distributions are seen for all three opioid receptors in relation to their putative endogenous ligands. Note also at these higher magnifications MOR1, DOR1, and KOR1 each has a distinct pattern of labeling in the dorsal horn (**B, D, F**): MOR1 labeling is preferentially seen in postsynaptic membranes, DOR1 staining is only seen in axon terminals, and KOR1 seems to label both pre- and postsynaptic structures. Scale bars = 250 μm (**A, C, E**); 25 μm (**B, B′, D, D′, F, F′**). Images **B, B′, D, D′, F, F′** are the results of projecting six optical sections taken at 0.4 μm intervals.

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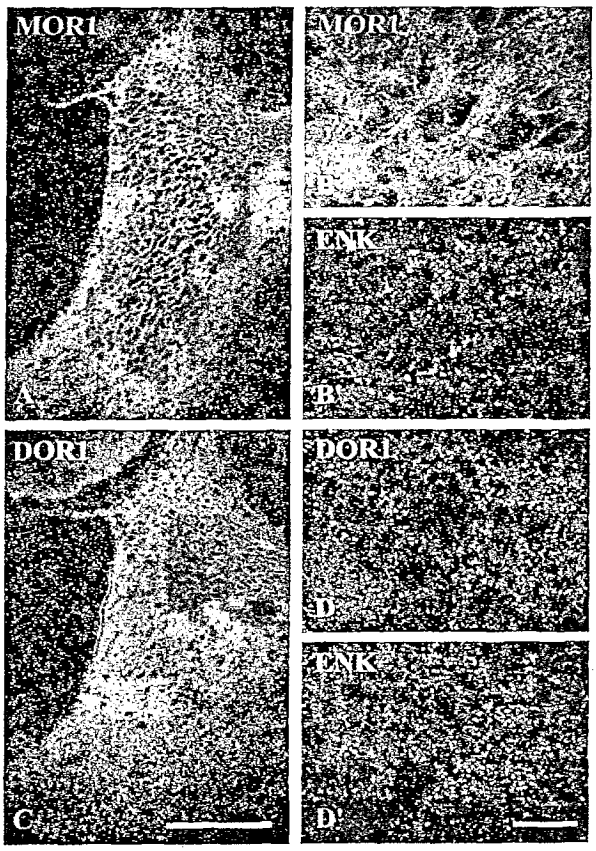

FIGURE 2. Localization of μ- and δ-opioid receptors and their putative endogenous ligand, enkephalin (ENK), in locus coeruleus as viewed with laser scanning confocal microscopy. Immunofluorescence images of coronal sections of the adult rat locus coeruleus single stained with rabbit anti-μ-opioid receptor (MOR1; **A**) or rabbit anti-δ-opioid receptor (DOR1; **C**). The opioid receptor immunoreactivity was visualized with cyanine 3.18-conjugated secondary antibodies. Note the different staining pattern for MOR1 and DOR1. To determine the relationship between ENK and MOR1 and DOR1, double-labeling immunofluorescence experiments using a mixture of either rabbit anti-MOR1 (**B**) and mouse anti-ENK (**B'**), or rabbit anti-DOR1 (**D**) and mouse anti-ENK (**D'**) were carried out. After incubation the sections were stained with lissamine rhodamine- and fluorescein-conjugated secondary antibodies. (**B–B'**) Dense staining of MOR1 was seen in the cell membrane of locus coeruleus neurons as well as in the neuropil. ENK-immunoreactive varicosities were present in close vicinity to MOR1-labeled structures; however, a mismatch in the relative abundance of ENK and MOR1 was observed. (**D–D'**) As in the case of the dorsal horn, DOR1-immunoreactivity was confined to varicose axonal profiles in locus coeruleus, suggesting a role as a presynaptic receptor. No unambiguous coexistence between DOR1 and ENK was seen, suggesting that DOR1 is not an autoreceptor for ENK in locus coeruleus. Scale bars = 200 μm (**A, C**); 25 μm (**B, B', D, D'**). Images **B, B', D, D'** are the results of projecting six optical sections taken at 0.4 μm intervals.

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Ventral Forebrain

Distinctive staining patterns for each of the three cloned opioid receptors are seen in the forebrain. Patches of MOR1-ir are scattered, especially in the dorsolateral aspect of the striatum (FIG. 4A), in a pattern that strongly resembles both μ-opioid binding and the location of MOR1 as determined by *in situ* hybridization.[6] DOR1-ir is not so prominent in the striatum, but is concentrated in irregular zones in the islands of Calleja (FIG. 4B), the substantia inominata, and the septal nuclei. KOR1-ir is also prominent in the ventral forebrain, especially in the vicinity of the islands of Calleja and the shell region of nucleus accumbens (FIG. 4C).

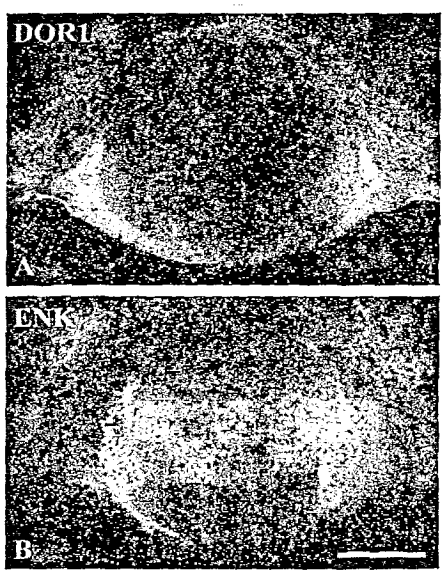

FIGURE 3. Spatial relations between δ-opioid receptor and its putative endogenous ligand, enkephalin, in the interpeduncular nuclei. Immunofluorescence images of a coronal section of the adult mouse interpeduncular nuclei as viewed with laser scanning confocal microscopy. The section was simultaneously incubated with a mixture of rabbit anti-δ-opioid receptor (DOR1, **A**) and mouse anti-enkephalin (ENK, **B**). The staining was visualized with lissamine rhodamine- and fluorescein-conjugated secondary antibodies. Note that the distribution of DOR1-immunoreactivity is found in a nucleus adjacent to the distribution of ENK-positive fibers, suggesting in this case that neurotransmission could possibly occur between nuclei. Scale bar = 250 μm (**A, B**).

Subcellular Localization of Opioid Receptors

DOR1 seems largely localized in axons and in vesicles undergoing axonal transport (FIG. 5). Surprisingly, neither DOR1-ir (in terminals in the superficial dorsal horn of the spinal cord) nor KOR1-ir (in terminals in the neural lobe of the pituitary) is prominent in the plasma membrane of nerve terminals. Rather, preliminary electron microscopic studies have demonstrated staining for these receptors is prominent in a population of large, clear vesicles (Arvidsson *et al.,* in preparation).

IMPLICATIONS OF FINDINGS

Some aspects of the neurotransmission accomplished by opioid peptides and their receptors have been thoroughly studied and thus serve as models of the more general phenomenon of peptidergic neurotransmission. In spite of these advances,

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FIGURE 4. Localization of μ-, δ-, and κ-opioid receptors in the forebrain as viewed with laser scanning confocal microscopy. Immunofluorescence images of coronal sections of the adult rat forebrain single stained with rabbit anti-μ-opioid receptor (MOR1; **A**), rabbit anti-δ-opioid receptor (DOR1; **B**) or rabbit anti-κ-opioid receptor (KOR1; **C**). The opioid receptor immunoreactivity was visualized with cyanine 3.18-conjugated secondary antibodies. (**A**) Intense MOR1 staining is seen in patches in the striatum. Note also weak labeling of the neuropil between the patches. (**B**) A relatively dense network of DOR1 positive fibers is seen in the area of the islands of Calleja. (**C**) Low-power micrograph of the ventral forebrain showing dense labeling of KOR1 in the shell of nucleus accumbens and in the olfactory tubercle including the islands of Calleja. Scale bars = 250 μm (**A**, **B**); 500 μm (**C**).

large gaps are apparent in our understanding of opioid neurotransmission. One of these gaps has been the lack of knowledge of the spatial relationships between nerve terminals that contain opioid peptides and the membranes that possess opioid receptors. A second, and related gap, has been the lack of identification of the endogenous opioid peptides that are likely to occupy a given opioid receptor subtype under physiological or pathophysiological circumstances.

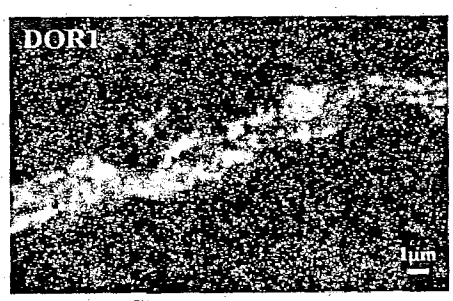

FIGURE 5. Intraaxonal localization of δ-opioid receptor (DOR1) as viewed with laser scanning confocal microscopy. High-magnification immunofluorescence image of a coronal section of the adult rat brainstem single stained with rabbit anti-δ-opioid receptor (DOR1) and visualized with cyanine 3.18-conjugated secondary antibodies. The DOR1 staining is seen in small, round structures with a size of approximately 200–300 nm. These labeled structures possibly represent vesicles transporting the receptor to its target (anterograde transport) or back to the cell (retrograde transport).

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By staining for both opioid peptides and opioid receptors we have been able to determine with cellular and subcellular precision the relationship between nerve fibers and terminals that contain opioid peptides and the receptors with which they are associated. From previous studies by others as well as our own studies, we believe that opioidergic neurotransmission is generally nonsynaptic.[10,11] We have found that the arrangement of the elements involved in opioidergic transmission falls between two extremes. First, as exemplified in the dorsal horn of the spinal cord and locus coeruleus, we observed a nonsynaptic interdigitation of nerve terminals containing opioid peptides with neuronal membranes bearing opioid receptors (with the interdigitations being separated by distances on the order of microns). Second, in some cases such as the subnuclei of the interpeduncular nucleus, ENK- and DOR-ir occur in entirely different subnuclei. Although this is a striking mismatch in the spatial distribution of this transmitter/receptor combination, the complementary and orderly relationship between ligand and receptor suggests the possibility that neurotransmission in this case may occur between nuclei (over distances on the order of hundreds of microns).

A further problem in opioid neurotransmission has been the confusion in assigning endogenous ligands to subtypes of opioid receptors.[44] Previous investigations of this problem used bioassays and radioligand binding to determine the rank order of potency of endogenous ligands for each subtype of opioid receptor. This approach has pharmacological validity, but depends greatly on the stability of the ligands tested. It is known that many neurotransmitters are very unstable in such preparations because of mechanisms that terminate the process of neurotransmission. An alternative, and more physiological approach to this problem is to determine the spatial relationship and relative abundance of each of the endogenous ligands to each of the receptor subtypes. The use of multicolor immunofluorescence studies in which both receptors and opioid peptides are localized simultaneously has greatly aided this task. Based on these studies, it appears that enkephalins are likely to be physiologically relevant ligands for both DOR1 and MOR1 in the superficial dorsal horn of the spinal cord and that dynorphin-related peptides are positioned to gain access to KOR1.

Little is known concerning the mechanisms that deliver newly synthesized receptor protein to the region of plasma membrane where it might be exposed to ligands. Autoradiographic studies suggest that some opioid receptors undergo axonal transport[42,45–47] and are likely to function at presynaptic sites.[48] One of the major surprises upon our initial immunocytochemical localizations of DOR1 was that it seems to be targeted almost exclusively to the axon of neurons, and thus it is most likely that it can act in a "presynaptic" manner. However, high magnification confocal and preliminary electron microscopic studies have suggested that a great fraction of DOR1 is not on the plasma membrane of axons or their terminals, but rather is found intra-axonally associated with vesicles. This suggests the interesting possibility that presynaptic opioid receptors may be stored, awaiting exteriorization, perhaps in response to stimulation (FIG. 6). If this proves to be the case, it suggests additional regulatory steps important in receptor function. Indeed, a recent report suggests that chronic morphine treatment reduces the rate of a slow phase of axonal transport, at least in mesolimbic dopamine neurons.[49] Together, these findings suggest that certain stimulus parameters and kinetics of receptor targeting might be important mechanisms underlying tolerance.

A further surprising aspect of the targeting of neuropeptide receptors was observed in dorsal root ganglion neurons, where it was observed that DOR1 and KOR1 are transported from the perikarya into the central and peripheral processes, whereas a portion of MOR1 remains in the perikarya and appears to decorate the

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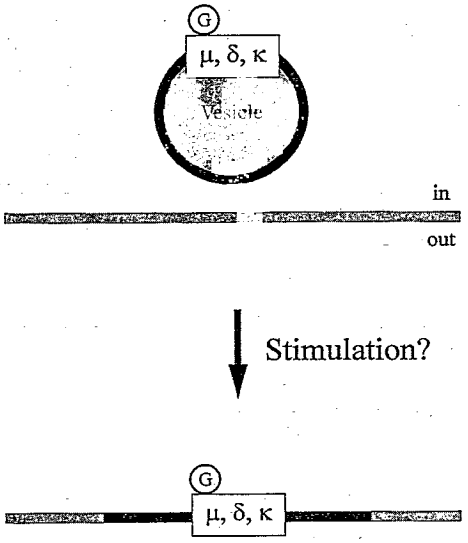

FIGURE 6. Schematic illustration of the hypothesis that presynaptic opioid receptors are inserted into the plasma membrane of the nerve terminal in an activity-dependent manner. Under normal conditions (*upper panel*) a major portion of opioid receptor immunoreactivity in nerve terminals is found associated with a distinct population of vesicles. Under conditions of increased activity (*lower panel*) the resultant fusion of the vesicle membrane with the plasma membrane of the nerve terminal results in the externalization of the receptor. G, heterotrimeric G-proteins.

plasma membrane of the perikarya.[6,50] Even more extreme, we recently determined that the Y1 receptor for neuropeptide Y is produced by a subset of dorsal root ganglion neurons, but it is almost completely retained by the perikaryon where it decorates the plasma membrane.[50] Although it has not been thought that signaling occurs within dorsal root ganglia, the position of MOR1 and the Y1 receptor suggest that blood-borne neuropeptides might gain access to ganglionic receptors, and thereby contribute to the regulation of activity of subsets of primary afferent neurons.

SUMMARY

The cloning of receptors for neuropeptides made possible studies that identified the neurons that utilize these receptors. *In situ* hybridization can detect transcripts that encode receptors and thereby identify the cells responsible for their expression, whereas immunocytochemistry enables one to determine the region of the plasma membrane where the receptor is located. We produced antibodies to portions of the predicted amino acid sequences of δ, μ, and κ opioid receptors and used them in combination with antibodies to a variety of neurotransmitters in multicolor immunofluorescence studies visualized by confocal microscopy. Several findings are notable: First, the cloned δ opioid receptor appears to be distributed primarily in axons, and therefore most likely functions in a presynaptic manner. Second, the cloned μ and κ opioid receptors are found associated with neuronal plasma membranes of dendrites and cell bodies and therefore most likely function in a postsynaptic manner. However, in certain, discrete populations of neurons, μ and κ opioid receptors appear to be distributed in axons. Third, enkephalin-containing terminals are often found in close proximity (although not necessarily synaptically linked) to membranes containing either the δ or μ opioid receptors, whereas dynorphin-containing termi-

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nals are often found in proximity to κ opioid receptors. Finally, a substantial mismatch between opioid receptors and their endogenous ligands was observed in some brain regions. However, this mismatch was characterized by complementary zones of receptor and ligand, suggesting underlying principles of organization that underlie long-distance, nonsynaptic neurotransmission.

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